Growth Regulating Proteins

Cross References to Related Applications

This application claims the priority of US provisional application no. 60/368,457 the disclosure of which is incorporated herein in its entirety.

Technical Field

The invention relates to a novel family of proteins having growth inhibiting activity and their diagnostic and therapeutical use.

Background Art

Growth is intimately linked not only to normal development but also to abnormal conditions such as tumorogenesis and cancer. In spite of its importance we still know relatively little about how growth is regulated at the cellular level, at the level of tissues and organs or at the level of the entire organism. Since growth is normally associated with cell multiplication much effort in understanding mechanisms regulating growth has focused on the mechanisms of cell cycle regulation. Indeed, our knowledge of how cells progress through the cell cycle has increased substantially (Nurse 2000). The preoccupation with cell division control has led to the assumption that growth is regulated by factors that control the cell cycle. Elegant experiments in Drosophila imaginal discs, however, reminded us old findings in yeast, that growth regulates the cell cycle and not vice versa (Nurse 1975). In the Drosophila experiments it was shown that accelerating the cell cycle time in clones of

cells did not stimulate net growth as measured by the area occupied by the clone, but produced more but smaller cells occupying the same area as the control clones. Conversely, slowing down the cell cycle by overexpression of the RB homolog RBF generated fewer but larger cells again occupying the same area (Neufeld, de la Cruz et al. 1998). Therefore, understanding of growth regulation during normal and abnormal development requires more than understanding cell cycle control. Understanding how growth is regulated at the cellular and tissue level will also provide novel approaches and targets for cancer therapy. Indeed, inhibitors that block cell growth such as Rapamycin are presently in clinical trial as anti-cancer drugs (Hidalgo and Rowinsky 2000). There is therefore an urgent need for means, which allow the diagnosis and the therapy of hyperproliferative diseases.

Disclosure of the Invention

Hence, it is a general object of the present invention to provide a protein comprising an ENTH domain and having growth inhibiting activity.

The term ENTH (Epsin NH_2 Terminal Homology domain) domain as used herein describes a conserved protein domain which was first found in proteins of the epsin family. Typically, this domain is located at the N-terminus of the proteins and said domain is characterised by 16 absolutely conserved residues: N-x(11-13)-V-x2-A-T-x(34-36)-R-x(7-8)-W-R-x3-K-x12-G-x-E-x15 -L-x11-12-D-x-G-R-x11-D-x7-R.

In a preferred embodiment said proteins lack a NPF domain which interacts with Eps15 and lack a DPW domain which binds to the clathrin adaptor AP2.

In a further preferred embodiment said proteins have an amino acid sequence which is at least 40%, preferably 50%, more preferably 60%, even more

preferably 80 % and most preferably 90% identical to the amino acid sequence set forth in Seq. Id. No. 2 (human ELP), or Seq. Id. No. 5 (hELP18aa) or Seq. Id. No. 4 (Drosophila ELP).

In a particular preferred embodiment said protein has an amino acid sequence which is identical to the amino acid sequence of Seq. Id. No. 2 (Homo sapiens), Seq. Id. No. 4 (Drosophila melanogaster) or Seq. Id. No. 5 (Homo sapiens h-ELP18aa).

Another object of the present invention are nucleic acid sequences encoding a protein of the present invention. In a preferred embodiment said nucleic acid sequence is selected from the group consisting of Seq. Id. No. 1 (h-ELP) and Seq. Id. No. 3 (dELP).

Another object of the present invention is a method of determining whether a subject, e.g. a human patient, is at risk for a disorder characterized by unwanted cell proliferation or aberrant control of differentiation.

Another aspect of the present invention provides antibodies and antibody preparations specifically reactive with an epitope of an epsin like protein.

A further object of the present invention is the use of proteins and/or nucleic acids of the present invention for gene therapy purposes.

In yet another aspect the present invention relates to the use of proteins and/or nucleic acids of the present invention for the treatment of hyperproliferative diseases such as e.g. cancer.

The present invention relates in a further aspect to a vector comprising a nucleotide sequence encoding a protein of the present invention. Typically, a vector comprises the regulatory sequences required to achieve expression in a host cell and it may contain necessary sequences required for plasmid replication in order to exist in an episomal state, or it may be

designed for chromosomal integration. The term regulatory sequence as used herein encompasses both the native regulatory sequence of a gene of the present invention and heterologous regulatory sequences.

Furthermore, the present invention provides a host cell transformed with a vector comprising a nucleotide sequence encoding a protein of the present invention. Any host cell being able to express a protein of the present invention can be used, e.g. bacteria, vertebrate and invertebrate cells. Said cells can e.g. be used for the production of epsin like proteins.

In a further aspect, the present invention relates to a method for the production of epsin like proteins wherein suitable host cells are transformed with a vector comprising a nucleic acid sequence encoding an epsin like protein, cultivation of said host cells under conditions allowing protein expression and isolation of the produced epsin like proteins. Any host cell being able to express a protein of the present invention can be used, e.g. bacteria, vertebrate and invertebrate cells.

Brief Description of the Drawings

The invention will be better understood and objects other than those set forth above will become apparent when consideration is given to the following detailed description thereof. Such description makes reference to the annexed drawings, wherein:

Figure 1A shows an electro scanning micrograph of a wild-type D. *melanogaster* eye as control;

Figure 1B shows an electro scanning micrograph of a compound eye largely composed of homozygous dELP mutant tissue. The eye displays a dramatic increase in size. The picture was taken from a female of the genotype y w ey-flp/y w; FRT82 $dELP^{4K2}$/FRT82 w+ c13R3;

Figure 2 shows a sequence alignment of dELP (Seq. Id. No 4) and hELP (Seq. Id. No 2). Dark boxes: identical residues. Grey boxes: conserved residues;

Figure 3 shows a Clustal W alignment of the 2 h-ELP isoforms: h-ELP (Seq. Id. No. 2) (sequence from the database) and h-ELP18aa (Seq. Id. No. 5) (sequence from EST). Identical amino acids are shown by asteriks below the alignment;

Figure 4A shows expression of h-ELP transcripts in human tissue and

Figure 4B shows expression of h-ELP transcripts in multiple cell lines. Northern blots (Clonetech) were hybridized with a 32P labeled h-ELP probe (nucleotides 751 to 1951). Hybridization was with ExpressHyb (Clonetech) at 68 °C with a final washing using 0.1 x SSC and 0.1 % SDS at 68°C for 10 minutes.

Figure 5 shows expression of hELP transcript: Cancer Profiling Array (BD Biosciences cat. 7841-1) membranes were hybridized with 5` labeled hELP probe (nucleotides 751 to 1951). Hybridization was performed at 68°c, according to manufacturer's recommendation with a final washing using 0.2 x SCC and 0.5% SDS at 68°C for 15 min. For sample reference see BD bioscience catalog.

Figure 6 shows the growth phenotypes caused by overexpression of delp in eye and wing in adult flies. A) Overexpression of dELP in the developing eye induces growth inhibiting phenotypes in the adult eye, whereas overexpression in the wing leads to a severe wing size reduction, which might also involve apoptotic effects as well. B) and D) delp UAS-lines as control. Genotypes: A) y w UAS-delp 3.15; eyGal4/+ B) y w UAS-delp 3.15; +/+ C) y w MS 1096/UAS-delp 3.15 D) y w 3.15 UAS-delp; +/+; MKRS/TM3.

Modes for Carrying out the Invention

To identify genes specifically involved in growth at a cellular, tissue and organismal level, the inventors took a genetic approach in Drosophila. They performed a genome-wide screen for recessive mutations that interfere or promote cell growth without affecting cell differentiation. These screens were carried out by taking advantage of a tissue-specific recombination system (Newsome, Asling et al. 2000) that generates genetically mosaic flies. These flies are homozygous for randomly induced mutations in the head tissue but heterozygous for the same mutation in the body and the germ line. Mutations in genes whose products selectively promote growth (potential oncogenes) will produce flies with small heads while mutations in genes whose products exert a growth inhibiting function (potential tumor suppressor genes) results in flies with larger than normal heads. The validity of this screen to find genes involved in tumorogenesis is exemplified by the identification of mutations in Drosophila homologues of known oncogenes as the Target of Rapamycin TOR, Myc, Ras causing a small-head phenotype and mutations in known tumor suppressor gene homologues like PTEN, LATS and TS1 causing a big-head phenotype ((Huang, Potter et al. 1999; Oldham, Montagne et al. 2000).

Mutations in the gene described in this invention produced flies with larger than normal heads (Fig 1) strongly suggesting an essential growth inhibiting function. The gene was later identified as being novel and named Epsin Like Protein (ELP)due to a common domain with the Drosophila Epsin protein. This domain, known as Epsin NH_2-Terminal Homology (ENTH) domain (Kay, Yamabhai et al. 1999), is found in all the Epsin family proteins (Rosenthal, Chen et al. 1999) which have been implicated in receptor mediated endocytosis and in the regulation of growth receptors (Carbone 1997;

Nakashima, Morinaka et al. 1999). However, ELP is missing two essential domains of Epsin involved in endocytosis: the C-terminal NPF domain which interacts with Eps15 (Chen, Fre et al. 1998) and the DPW central motif which binds to the clathrin adaptor AP2 (Robinson PJ, Liu JP,. Trends in neuroscience 1994) and Clathrin (Rosenthal, Chen et al. 1999). Since the three dimensional structure of the ENTH domain resembles the one of the Armadillo repeat, it has been postulated that this domain might also mediate nucleo-cytoplasmic shuttling (Hyman, Chen et al. 2000; Vecchi, Polo et al. 2001).

The ENTH domain has been described to bind phosphatidylinositol-4,5-bis phosphate (PIP2) (PIP2) (Itoh, Koshiba et al. 2001), the dephosphorylated form of the membrane phospholipid PI(3,4,5)P3, which plays a pivotal role in the signal propagation from membrane receptors to downstream components (Martin 2001). Thus, in analogy to proteins containing a PH domain, known to bind to and be regulated by PIP3 levels, the localization and the function of ENTH domain containing proteins might be regulated by PIP2 levels.

The fact that the growth phenotype observed in cells lacking the tumor suppressor gene LATS is very similar to the one of cells homozygous for mutations in the gene described herein indicates that ELP may also constitute a tumor suppressor gene.

In the scope of the present invention the D. melanogaster ELP protein and two human ELP isoforms were identified as well as the corresponding genes.

A first aspect of the present invention relates to espin like proteins (ELP) which are characterised by the presence of an ENTH domain and their growth inhibiting activity. In the scope of the present invention the epsin like proteins of Drosopila melanogaster and humans have been cloned and characterised.

It could be shown that dELP overexpression induces cell death in the fly wing and strongly reduce the size of the compound eye (again an indication of its negative effect on growth) and that overexpression of hElp and dElp transgenes in transheteroallelic mutant D. melanogaster background partially rescues homozygous mutant lethality from late larval to pupal stages. These findings prove that the drosophila and the human protein have the same function.

The growth inhibiting activity of said proteins indicates that the epsin like genes function as tumor suppressors.

Included within the term ELP protein are also functional fragments, variants or derivatives of any of the proteins defined hereinbefore. The proteins of the present invention can be provided as chimeric proteins for example as recombinant fusion proteins.

A "chimeric protein" or "fusion protein" is a fusion of a first amino acid sequence encoding one of the subject ELP polypeptides with a second amino acid sequence defining a domain foreign to and not substantially homologuous with any domain of one of the ELP proteins. A chimeric protein may present a foreign domain which is found (albeit in a different protein) in an organism which also expresses the first protein, or it may be an "interspecies", "intergenic", etc. fusion of protein structures expressed by different kinds of organisms. In general, a fusion protein can be represented by the general formula X-ELP-Y, wherein ELP represents a portion of the protein which is derived from one of the ELP proteins, and X and Y are independently absent or represent amino acid sequences which are not related to one of the ELP sequences in an organism, including naturally occurring mutants.

In a second aspect the present invention relates to nucleic acid sequences encoding ELP proteins. DNA sequence polymorphisms that do lead to changes in the

amino acid sequence of ELP are also comprised by the present invention. One skilled in the art will appreciate that these variations in one or more nucleotides (up to about 3-5% of the nucleotides) of the nucleic acids encoding polypeptides having an activity of an ELP polypeptide may exist among individuals of a given species due to natural allelic variation. Fragments of the nucleic acids encoding an active portion of the epsin like proteins are also within the scope of the invention. As used herein, an *elp* gene fragment refers to a nucleic acid having fewer nucleotides than the nucleotide sequence encoding the entire amino acid sequence of ELP represented in Seq. Id No:2, Seq. Id No:4 or Seq. Id. No. 5, yet preferably encodes a peptide which retains some biological activity of the full length protein or regains some biological activity in the presence of a suitable agonist/antagonist. Nucleic acid fragments within the scope of the present invention include those capable of hybridizing under high or medium stringency conditions with nucleic acids from other species for use in screening protocols to detect and isolate other *elp* alleles and/or homologs, as well as those capable of hybridizing with nucleic acids from human specimens for use in detecting the presence of a nucleic acid encoding an ELP protein, including alternate isoforms, e.g. mRNA splicing variants. Nucleic acids within the scope of the invention may also contain linker sequences, modified restriction endonuclease sites and other sequences useful for molecular cloning, expression or purification of recombinant forms of the subject dispatched polypeptides.

In a further aspect the present invention relates to a method for the determination whether a subject, e.g. a human patient, is at risk for a disorder characterized by unwanted cell proliferation or aberrant control of differentiation. The method includes detecting, in a tissue sample of the subject, the presence or absence of a genetic lesion characterized by

at least one mutation in an epsin like gene or the mis-expression of an epsin like gene.

To illustrate, nucleotide probes can be generated from the genes of the present invention which facilitate histological screening of intact tissue and tissue samples for the presence (or absence) of ELP encoding transcripts. The use of probes directed to ELP messages, or to genomic ELP sequences, can be used for both predictive and therapeutic evaluation of allelic mutations which might be manifest in, for example, neoplastic or hyperplastic disorders (e.g. unwanted cell growth). The oligonucleotide probes can help facilitate the determination of the molecular basis for a developmental disorder which may involve some abnormality associated with expression (or lack thereof) of an epsin like protein. For instance, variation in polypeptide synthesis can be differentiated from a mutation in a coding sequence.

E.g. by dot-blot experiments comparing ELP RNA expression in tumor tissue vs. normal tissue, significant down-regulation of ELP RNA could be found in connection with several cancers, in particular lung cancer samples, kidney cancer samples and stomach cancer samples.

In preferred embodiments, the diagnostic method can be characterized as comprising: detecting, in a tissue sample of the subject (e.g. a human patient), the presence or absence of a genetic lesion characterized by at least one of (i) a mutation of a gene encoding an epsin like protein or (ii) the mis-expression of an epsin like protein. To illustrate, such genetic lesions can be detected by ascertaining the existence of at least one of (i) a deletion of one or more nucleotides from a gene encoding an epsin like protein, (ii) an addition of one or more nucleotides to a gene encoding an epsin like protein, (iii) a substitution of one or more nucleotides of a gene encoding an epsin like protein, (iv) a gross

chromosomal rearrangement of a gene encoding an epsin like protein, (v) a gross alteration in the level of a messenger RNA transcript of a gene encoding an epsin like protein, (vi) the presence of a non-wild type splicing pattern of a messenger RNA transcript of a gene encoding an epsin like protein, (vii) a non-wild type level of an epsin like protein and (viii) a mutation in the 5' untranslated region or 3' untranslated region of an elp gene.

In one aspect of the invention there is provided a probe/primer comprising an oligonucleotide containing a region of nucleotide sequence which is capable of hybridizing to a sense or antisense sequence of SEQ ID No:1, or naturally occurring mutants thereof, or 5' or 3' flanking sequences or intronic sequences naturally associated with a gene of the present invention. The probe is exposed to nucleic acid of a tissue sample; and the hybridization of the probe to the sample nucleic acid is detected. In certain embodiments, detection of the lesion comprises utilizing the probe/primer in a polymerase chain reaction (PCR) (see, e.g., U.S.Pat. No. 4,683,195 and 4,683,202) or, alternatively, in a ligation chain reaction (LCR) (Landegren, Kaiser et al. 1988; Nakazawa, English et al. 1994) the later of which can be particularly useful for detecting point mutations in genes. Alternatively, immunoassays can be employed to determine the level of proteins.

In a preferred embodiment the mutation in the *elp* gene is located within the ENTH domain encoding region, in the 5' untranslated region of the elp gene or in the gene region right adjacent to the ENTH domain.

Another aspect of the present invention provides antibodies and antibody preparations specifically reactive with an epitope of an epsin like protein.

For example, by using immunogens derived from ELP proteins, e.g. based on the cDNA sequences, anti-protein/anti-peptide antisera or monoclonal antibodies can be made by standard protocols (See, for example, (Harlow and Lane 1988)). A mammal, such as a mouse, a hamster or rabbit can be immunized with an immunogenic form of the peptide (e.g., a vertebrate dispatched polypeptide or an antigenic fragment which is capable of eliciting an antibody response). Techniques for conferring immunogenicity on a protein or peptide include conjugation to carriers or other techniques well known in the art. An immunogenic portion of an ELP protein can be administered in the presence of adjuvant. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other immunoassays can be used with the immunogen as antigen to assess the levels of antibodies.

In a preferred embodiment, the antibodies are immunospecific for antigenic determinants of an ELP protein of a vertebrate organism, such as a mammal. Following immunization of an animal with an antigenic preparation of an ELP protein, anti-ELP antisera can be obtained and, if desired, polyclonal anti-ELP antibodies isolated from the serum. To produce monoclonal antibodies, antibody-producing cells (lymphocytes) can be harvested from an immunized animal and fused by standard somatic cell fusion procedures with immortalizing cells such as myeloma cells to yield hybridoma cells. Such techniques are well known in the art, and include, for example, the hybridoma technique (originally developed by (Kohler and Milstein 1975)), the human B cell hybridoma technique (Kozbar 1983), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole 1985). Hybridoma cells can be screened immunochemically for production of antibodies specifically reactive with an ELP protein and monoclonal antibodies isolated from a culture comprising such hybridoma cells.

The term antibody as used herein is intended to include fragments thereof which are also specifically reactive with one of the ELP polypeptides. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner as described above for whole antibodies. For example, $F(ab)_2$ fragments can be generated by treating antibody with pepsin. The resulting $F(ab)_2$ fragment can be treated to reduce disulfide bridges to produce Fab fragments. The antibody of the present invention is further intended to include bispecific and chimeric molecules having affinity for an ELP protein conferred by at least one CDR region of the antibody.

Both monoclonal and polyclonal antibodies (Ab) directed against ELP polypeptides, or ELP variants, and antibody fragments such as Fab and $F(ab)_2$, can be used to block the action of one or more ELP proteins and allow the study of the role of these proteins in, for example, embryogenesis and/or maintenance of differential tissue. In a similar approach, hybridomas producing anti-ELP monoclonal antibodies, or biodegradable gels in which anti-ELP antibodies are suspended, can be implanted at a site proximal or within the area at which ELP action is intended to be blocked. Experiments of this nature can aid in deciphering the role of this and other factors that may be involved in growth regulation. The antibodies are as well suitable as tools for the use in diagnostic assays for the identification of diseases associated with ELP proteins or a predisposition thereof e.g. by detecting reduced or enhanced Elp protein expression, by detecting a cellular mislocalisation of an ELP protein or by detecting aberrant ELP proteins in a tissue or body fluid sample e.g. blood, of an individual. Said aberrant forms of ELP protein can e.g. be the result of an incomplete or different splicing and lead to shorter or longer ELP proteins with enhanced or reduced or different activity than the wildtype ELP protein. Furthermore, said

aberrant ELP protein forms can be chimeric proteins wherein full length ELP protein or fragments thereof are fused to another protein or fragments thereof. Said chimeric protein can e.g. have arisen from a deletion between two genes or be the result of a genomic rearrangement. The man skilled in the art knows suitable methods for the detection of proteins using antibodies in a tissue and/or body fluid sample. Said methods include but are not limited to Western blots, Enzyme Linked Immunosorbent Assay ("ELISA"), cell-based ELISA, immunoprecipitations, slot or dot blots, radioimmunoassays, and fluorescent immunoassays.

In another aspect the present invention relates to the use of proteins and/or nucleic acids of the present invention (whether full-length or a desirable fragment) for the gene therapy of hyperproliferative diseases associated with ELP. An appropriate vector for delivery may be readily selected by one of skill in the art. Gene therapy vectors are readily available from a variety of sources, and include, e.g., adeno-associated virus [International patent application No. PCT/US91/03440], adenovirus vectors (Ishibashi, Brown et al. 1993; Kay, Landen et al. 1994), or other viral vectors, e.g., various poxviruses, vaccinia, etc. Methods for insertion of a desired gene, e.g., BAP-1, and obtaining in vivo expression of the encoded protein, are well known to those of skill in the art.

The proteins and/or nucleic acids of the present invention are as well suitable for the treatment of hyperproliferative diseases e.g. in the form of a pharmaceutical preparation. The pharmaceutical preparations can - if desired - be mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, coloring, flavoring and/or aromatic substances and the like which do not deleteriously react with the active compounds.

The proteins may be administered by injection (i.e., subcutaneous, intravenous, intramuscular, intratumoral, intraperitoneal, preferably intramuscular), oral administration, inhalation, transdermal application, topical administration or rectal administration, preferably by injection, transdermal or topical administration. Depending on the route of administration, the peptides in the pharmaceutical compositions may be coated in a material to protect them from the action of certain enzymes. A person skilled in the art would be familiar with the coating which would be suitable for delivery of the peptide to a particular site. Organic substances may also be included in the compositions to prolong the pharmacologic actions of the peptides. Examples of such organic substances include non-antigenic gelatin, carboxymethylcellulose, sulfonate or phosphate ester of alginic acid, dextran, polyethylene glycol and other glycols, phytic acid, polyglutamic acid, and protamine.

For therapy purposes, an active ELP peptide or a fragment thereof can as well be administered in form of a conjugate with an appropriate carrier molecule. The carrier molecule allows an enhanced cellular uptake of the ELP peptide into the cell and/or the delivery of the ELP peptide to specific target cells e.g. tumor cells. Suitable carrier molecules are e.g. transferrin or viral cell-entry proteins.

The invention relates furthermore to antisense molecules which can be used to down-regulate expression of ELP molecules in cells. The anti-sense reagent may be antisense oligonucleotides (ODN), particularly synthetic ODN having chemical modifications from native nucleic acids, or nucleic acid constructs that express such anti-sense molecules as RNA. The antisense sequence is complementary to the mRNA of the targeted gene, and inhibits expression of the targeted gene products. Antisense molecules inhibit gene

expression through various mechanisms, e.g. by reducing the amount of mRNA available for translation, through activation of RNAse H, or steric hindrance. One or a combination of antisense molecules may be administered, where a combination may comprise multiple different sequences.

Antisense molecules may be produced by expression of all or a part of the target gene sequence in an appropriate vector, where the transcriptional initiation is oriented such that an antisense strand is produced as an RNA molecule. Alternatively, the antisense molecule is a synthetic oligonucleotide. Antisense oligonucleotides will generally be at least about 7, usually at least about 12, more usually at least about 20 nucleotides in length, and not more than about 500, usually not more than about 50, more usually not more than about 35 nucleotides in length, where the length is governed by efficiency of inhibition, specificity, including absence of cross-reactivity, and the like. It has been found that short oligonucleotides, of from 7 to 8 bases in length, can be strong and selective inhibitors of gene expression (see (Wagner, Matteucci et al. 1996)).

Another aspect of the invention relates to post transcriptional silencing of *elp* genes by means of RNA interference (RNAi). RNAi is a form of post-transcriptional gene silencing mediated by short double stranded RNAs (dsRNA) that has been described in plants, nematode, invertebrates organisms and mammalian cell culture ((Ngo, Tschudi et al. 1998) (Vaucheret and Fagard 2001) (Kennerdell and Carthew 1998; Caplen, Fleenor et al. 2000; Elbashir, Harborth et al. 2001; Timmons, Court et al. 2001). Double stranded RNAs (dsRNA) have been shown to induce a degradation response in which single stranded RNA complementary to the short dsRNA is rapidly degraded (Montgomery and Fire 1998; Montgomery, Xu et al. 1998). RNAi can thus be used to reduce gene expression for instance in whole organisms or invertebrate and

vertebrate cell lines (Kennerdell and Carthew 1998; Caplen, Fleenor et al. 2000; Clemens, Worby et al. 2000; Elbashir, Harborth et al. 2001). ELP dsRNA can be made from cDNA or genomic DNA templates, as long as most of the dsRNA corresponds to exon regions. Normally, target regions of 700 to 800 base pair are the most active. However, it is known that dsRNAs as short as 200 base pair and as long as 2000 base pairs have potent interfering activities. Both RNA strands can be synthesized simultaneously from a PCR fragment, which contains for instance a T7, SP6 or a T3 promoter on each end. This PCR fragment can be generated by amplification of ELP cDNA or genomic DNA with 2 primers containing e.g. T7-polymerase binding sites. The PCR reaction can then be performed with a suitable template containing ELP sequences. Taq polymerase gives the best yields, but another polymerase like Pfu may be used, too. The first 10 cycles should have a 40°C annealing step, followed by 35 cycles with a 55°C annealing step. DMSO can be added to a final concentration of 5% when needed. Phenol-chloroform extract and ethanol precipitation in NH_4OAc may be used to isolate the PCR template from the reaction mix however other commercially available PCR-purification kit can be used as well. The RNA synthesis reaction can be performed in 50 µl volume with 1 µg of PCR DNA template using an appropriate RNA polymerase. The MEGAscript™ kits from Ambion work very well. The RNA becomes double-stranded during the synthesis reaction. The DNA template can be removed with RNase-free DNAase and the dsRNA can be purified by phenol-chloroform extraction and ethanol precipitation. Typical yields of RNA from 1 µg DNA template are in the 80 to 120 µg range. dsRNA can be stored as a NaOAc/ ethanol precipitate at -80°C until immediately before use.

The quality of the dsRNA can be monitored by native agarose gel electrophoresis in TBE. Only preparations should be used in which the electrophoretic

mobility of most of the RNA is shifted to the mobility expected for dsRNA (very close to duplex DNA mobility) of the appropriate length. To reduce ELP expression in mammalian cells, short 20-23mer dsRNA should be preferentially used (Elbashir, Harborth et al. 2001; Elbashir, Lendeckel et al. 2001). Such short dsRNA have a 3'-overhang as described in (Elbashir, Harborth et al. 2001) and can be synthesized *in vitro* by standard methods.

Several methods to introduce dsRNA into cells can be found in the literature and said methods are known to a man skilled in the art.

The invention is now further illustrated by means of examples.

These examples are provided merely as illustrative of various aspects of the invention and should not be considered to limit the invention in any way.

Experimental part

To identify genes specifically involved in growth at a cellular, tissue and organismal level, the inventors took a genetic approach in Drosophila. They performed a genome-wide screen for recessive mutations that interfere or promote cell growth without affecting cell differentiation. These screens were carried out by taking advantage of a tissue-specific recombination systems (Newsome, Asling et al. 2000) that generates genetically mosaic flies. These flies are homozygous for randomly induced mutations in the head tissue but heterozygous for the same mutation in the body and the germ line. Mutations in genes whose products selectively promote growth (oncogenes) will produce flies with small heads while mutations in genes whose products exert a growth inhibiting function (tumor suppressor genes)

results in flies with larger than normal heads. The validity of this screen is exemplified by the identification of mutations in the gene coding for the Target of Rapamycin (TOR) causing a small-head phenotype and mutations in the gene encoding the tumor suppressor gene PTEN causing a big-head phenotype (Huang, Potter et al. 1999; Oldham, Montagne et al. 2000).

Example I: Pinhead screening

Mutations in components of the insulin receptor signaling pathway impair cellular growth in a cell-autonomous fashion (Bohni, Riesgo-Escovar et al. 1999; Verdu, Buratovich et al. 1999; Weinkove, Neufeld et al. 1999; Brogiolo, Stocker et al. 2001). Clones of mutant cells bear a severe growth disadvantage and remain small compared to their wild-type sister clones. If mitotic recombination is forced to occur by the constant supply of flp recombinase and the sister clone gets eliminated by means of a cell-lethal mutation, these clones can, however, cover substantial fractions of whole organs. Driving the expression of the flp recombinase under the control of eyeless regulatory sequences results in the formation of tissue-specific clones in the eye imaginal disc solely. In combination with a cell-lethal mutation on the homologous chromosome, this system allows for the generation of eyes and head capsules largely composed of cells that are homozygous mutant for the gene of interest (Newsome, Asling et al. 2000). Such mosaic flies that lack the function of the IRS homolog Chico or of the insulin receptor (Inr) specifically in the descendants of the eye imaginal disc show a very characteristic phenotype. While their bodies are of normal size, their eyes and heads are dramatically reduced (Bohni, Riesgo-Escovar et al. 1999; Brogiolo, Stocker et al. 2001). In order to identify mutations in

growth-modulating genes based on similar phenotypes, males carrying target sites for the flp recombinase near the base of the right arm of the 3rd chromosome (82FRT) were subjected to EMS mutagenesis and crossed to females that brought in four elements: the source of the recombinase (ey-flp), FRT sites at the corresponding position, a dominant eye marker (w+) and a cell-lethal mutation (c13R3). In the mosaic flies of the F1 generation, the effects of homozygosity for newly induced mutations on 3R can be observed in the heads and the eyes. The presence of homozygous mutant tissue can easily be visualized by the loss of the pigment marker w+ resulting in white eye tissue.

Screening of mosaic flies

Males carrying FRT sites on the right arm of chromosome 3 (FRT82; (Xu and Rubin 1993)) were fed with 33mM EMS according to standard protocols and crossed to females of the genotype y w ey-flp; FRT82 w+ c13R3/TM6B y+. c13R3 is a recessive cell-lethal mutation that has been generated on the FRT82 w+ chromosome (Newsome, Asling et al. 2000). Half of the F1 progeny was of the genotype y w ey-flp/+ or Y; FRT82 */FRT82 w+ c13R3 and was scored for eyes and heads of abnormal size. Positives were re-crossed to y w ey-flp; FRT82 w+ c13R3/TM6B y+ to check for germline transmission. About 50'000 mosaic flies were screened to reach saturation, and 69 mutations that caused a big head phenotype were established.

Complementation group analysis

52 of the 69 mutations causing a big head phenotype fell into nine complementation groups. A complementation group is defined by a number of at least two alleles which in any pairwise combination fail to complement the lethality associated with homozygosity of each of the alleles. Mutations belonging to four of these complementation groups resulted in a hyperproliferative

phenotype: the supernumerary ommatidia caused the formation of folds that gave the eyes a tumorous appearance (Figure 1, right panel).

Meiotic and SNP mapping

One of the four complementation groups showing a hyperproliferative phenotype consisted of four alleles. One representative allele was mapped meiotically using following genetic markers: a mini-w+ bearing P-element at cytological position 87E, a w+ bearing P-element at 90E, and a y+ bearing P-element at 96E ((Xu and Rubin 1993)). The rough map position was confirmed and refined by complementation analysis using deficiencies within the same region. More restrictive mapping was done by assessing the frequency of recombination between alleles and nearby marked P-element insertions. The P-elements used for the mapping were EP(3)0738 (94A1-2), l(3)j5B5 (94A1-2) and l(3)L3560 (94A5-7). In this manner the genetic position of the mutations could be narrowed down to chromosome position 94A in close proximity to the P-element insertion l(3)L3560.

The genetic analysis positioned the site of mutation in a 300 kbp interval of roughly 120 kb within scaffold AE003738 (nucleotides 344000 - 464000). To specifically determine the molecular site of the mutation recombinants were mapped using SNPs as molecular markers in that specific region. SNPs were resolved by denaturing HPLC analysis using the WAVE system ((Underhill, Jin et al. 1996)). The site of mutation was narrowed down to an interval of 30 kbp. The precise location of a mutation was identified by analysis of candidate genes using the WAVE system and subsequent sequencing.

Example II: Expression of ELP in Drosophila melanogaster

A specific transgene can be expressed in Drosophila in the whole organism, in a particular organ or in a specific cell type, during the whole life or only at a specific developmental stage, and at different levels. An overview of the standard methods used in Drosophila genetics can be found in [Brand, 1993; Perrimon, 1998; Perrimon, 1998]. As a putative tumor suppressor gene, overexpression of wild-type or mutant ELP protein is expected to interfere with growth. To proof this hypotesis, transgenic flies carrying UAS transgenes encoding wild-type Drosophila ELP protein were crossed with flies having Gal4 expressed under the control of different tissue-specific promoters (Figure 6 and Table 2). The GAL4-driven constructs include but are not limited to eyeless-Gal4, vestigal-Gal4, MS1096-Gal4 (Capdevila and Guerrero 1994). In a similar way, it is possible to test the function of mutants proteins with amino acid substitutions or internal deletions.

	GMR*Gal4*	ey*Gal4*	C765*Gal4*	MS1096	ap*Gal4*	arm*Gal4*	da*Gal4*
3.15 on X	rough eye	vs eye omm #↓	subviable, +/- veins	subviable; ru. wings	lethal	no vis. ph.	lethal
3.24 B on IIIrd	rough eye	vs eye omm #↓	subviable, +/- veins	subviable; ru. wings	lethal	no vis. ph.	lethal

Table 2. Overexpression of DELP in different tissues. *Transgenic animals bearing two independent insertions of delp were crossed to respective GAL4-sources indicated in the first row to overexpress dELP in a variety of tissues. Abbreviations: vs eye omm #↓: very small eyes, ommatidia number reduced; +/- veins: additional or lost veins in wing; ru. wings: rudimentary wings, very small in size; no vis. ph.: no visible phenotype*

With the visible phenotype obtained by overexpressing wild-type (as in Figure 6) or likely mutant ELP, this system can be used to perform structure/function analysis of the ELP protein. Such a phenotype can also be used to screen for dominant or recessive loss of function mutations in other genes resulting in a suppression of the phenotype. Alternatively, using the Enhancer-Promoter (EP) Element (Rorth 1996)a screen for genes that supress the phenotype when they are overexpressed can be conducted. In this case, overexpression of a random gene is caused by the integration of an EP element into the 5' end of this gene.

Example III: Cloning of the human homologue

Human ELP (hELP) (Seq. Id. No. 1,2,5) was identified by searching the public sequence database for predicted coding sequences and expressed sequence tags (ESTs) having homology to the sequence of dELP using the program Blastp (http://www.ch.embnet.org/software/aBLAST.html). dELP showed statistically significant similarity to a predicted gene on chromosome V and to several ESTs. The hELP full-length cDNA was obtained from the full-length human cDNA clone collection from Resgen (Accession AL529948 Clone ID CS0DD005YJ09 Library LTI_NFL001_NBC4). This collection of cDNAs was derived from libraries constructed using oligodT primer and Superscript II reverse transcriptase. After the assembly process, the sequence was verified by crosschecking with genomic DNA sequences and the publicly available data.

To confirm the functional homology between Drosophila and human ELP, human and Drosophila full-length ELP cDNA were cloned into a Drosophila expression vector like the pUAS transformation vector (Phelps and

Brand 1998). Transgenic flies can be generated according to the method described in (Basler and Hafen 1988). The transgenes crossed into flies that are transheterozygous for anyone of two *elp* mutant alleles and also contain a Gal-4 transgene allowing ubiquitous or tissue and/or stage-specific expression of the UAS-transgenes. The Gal4 transgenes include but are not limited to the actin-Gal4, tubulin-Gal4, and heatshock-Gal4. As shown in Table 1 ubiquitous expression of Drosophila or the human transgene did at least partially rescue the lethality associated with the transheterozygosity of the *elp* mutant alleles from late larval to pupal stages. This demonstrates that the *help* gene is the functional homologue of the Drosophila *elp* gene (see Figure 6).

genotype abbreviated	Rescue at 25 °C	Rescue at 18 °C
5.20d/hs*Gal4*; $Delp^{1U1}/Delp^{2W2}$	non *Tb* pupae	-
2.4a/arm*Gal4*; $Delp^{1U1}/Delp^{2W2}$	1 non *Tb* pupa	no progeny
2.4a/hs*Gal4*; $Delp^{1G1}/Delp^{4K2}$	small non *Tb* pupae	non *Tb* pupae
2.4a/Act5C*Gal4*; $Delp^{1U1}/Delp^{2W2}$	very few offspring	-
5.20d/arm*Gal4*; $Delp^{1G1}/Delp^{4K2}$	small non *Tb* pupae	-
5.20d/arm*Gal4*; $Delp^{1U1}/Delp^{2W2}$	small non *Tb* pupae	small non *Tb* pupae
5.20d/Act5C*Gal4*; $Delp^{1G1}/Delp^{4K2}$	MKRS/*elp y*$^+$ appear	MKRS/*elp y*$^+$ appear

Table 1. Rescue analysis of *delp*-alleles by ubiquitous ELP overexpression. UAS*Elp*-transgenes were used to overexpress Elp in transheteroallelic homozygous mutant situation. UAS-*Elp*-transgenes used: 2.4a: UAS-*hElp*18aa; 5.20d: UAS-*DElp*-ENTH. *Elp*-alleles used: $Delp^{1G1}$: V266F-mutation; $Delp^{4K2}$: E27G-mutation; $Delp^{1U1}$: transition in 5'UTR; $Delp^{2W2}$: transition in 5'UTR. GAL4-sources used: arm*Gal4*: armadillo-*Gal4*; hs*Gal4*: heatshock-*Gal4*; Act5C*Gal4*: Actin5C-*Gal4*. UAS-transgenes and *Gal4*-strains were marked by w^+.

Alternatively, the Drosophila and human full-length cDNA can be inserted in the following sequence in a transformation vector containing a tubulin promoter: tubulin promoter - FRT - cDNA STOP - FRT - Gal4 (abbreviated: tub:>cDNA>Gal4). In this way, the rescue transgene is directly controlled by the tubulin promoter resulting in lower and possibly more physiological levels of ELP expression than with the Gal4 system. Furthermore, in an ELP mutant background the *elp* cDNA can be excised in clones of cell by expressing the FLP recombinase either under a heat-shock inducible promoter of under a

tissue specific promoter. The mutant cells generated in this way can be recognized by means of a UAS-GFP reporter since in these cells GAL4 is driven by the tubulin promoter. This method has been recently described by Kramps et al. (Cell 109: 47-60, 2002).

Example IV: Use of ELP DNA as a hybridization probe

The following method describes use of a non-repetitive nucleotide sequence of *elp* as a hybridization probe. The method can be applied to screen for ELP homologs as well. DNA comprising the sequence of *elp* (as shown in Seq. Id. No. 1 and 3) is employed as probe to screen for homolog DNAs (such as those included in cDNA or genomic libraries).

Hybridization and washing of the filters containing either library DNAs is performed under standard high stringency conditions (Sambrook, Fritsch et al. 1989). Positive clones can be used to further screen larger cDNA library platings. Representative cDNA-clones are subsequently cloned into pBluescript (pBS, Stratagene) or similar cloning vectors, and sequenced.

Example V: Use of elp as a hybridization probe for in situ hybridization.

In situ hybridization of Drosophila *elp* mRNA can be performed in embryo as described in (Tautz and Pfeifle 1989). However, with small modifications it can also be used to detect any mRNA transcript in Drosophila or vertebrate tissue sections. Labeled RNA probes can be prepared from linearized *elp* cDNA, or a fragment thereof, e.g. using the DIG RNA labeling Kit (SP6/T7) (Boehringer Mannheim) or ^{32}P-labeling following the manufacturer's recommendations. A similar method can be used with help

as a hybridization probe to screen human tissues (see Figure 4, and 5).

Example VI: Expression of ELP proteins in E. coli

The following method describes recombinant expression of ELP in bacterial cells. Alternatively, recombinant proteins can be produced and isolated from insect and mammalian cells (Sambrook, Fritsch et al. 1989). DNA encoding full-length or a truncated ELP form is fused downstream of an epitope tag or glutathione-S-transferase (GST) cDNA and a thrombin cleavage site contained within an inducible bacterial expression vector. Such epitope tags include poly-his, S-protein, thioredoxin, and immunoglobin tags. A variety of plasmids can be employed, including commercially available plasmid such as pGEX-4T (Pharmacia).

Briefly, a bacterial expression plasmid containing the ELP sequence, for instance fused to a GST-sequence is transformed by conventional methods into protease deficient E.coli such as BL21 (e.g. Stratagene). A bacterial colony containing the plasmid is then expanded overnight in selection medium to reach saturation. The next morning, this culture is diluted 1:100 and bacterial are allowed to grow to an optical density (OD600) of 0.6. Protein production is initiated by addition of an inducer of the promoter under which GST-ELP fusion protein is expressed. A variety of inducers can be employed depending on the expression vector used, including IPTG.

Expressed GST tagged ELP can then be purified, for instance, using affinity beads or affinity chromatography, such as glutathione beads (commercially available e.g. from Pharmacia). Extracts are prepared by lysing the Lgs-expressing bacteria in sonication buffer

(10 mM Tris HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 1.5% sarkosyl, 2% Triton-X-100, 1 mM DTT and protease inhibitors), followed by short sonication on ice (e.g. 3 times 20 seconds at middle power) and centrifugation. Cleared supernatants are then incubated under gentle rotation for example with glutathione beads for 1 hrs at 4°C. Next beads are washed several time in washing buffer (20 mM Tris pH 8.0, 200 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM MgCl2, 0.5% NP40), and finally stored in storage buffer (20 mM Tris pH 8.0, 200 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM MgCl2, 10 % glycerol, 0.5% NP40, and proteinase inhibitors). Alternatively, a His-tagged or IgG tagged ELP can be purified using Ni2+-chelate affinity chromatography or Protein A or Protein G column chromatography, respectively.

The quality of the preparations can be checked e.g. by SDS-gel electrophoresis and silver staining or Western blot.

In case the epitope tagged has to be cleaved, several methods are available depending on the presence of a cleavage site between the epitope tagged and the ELP protein. For example, it is possible to produce a GST-ELP fusion protein containing a thrombin cleavage site right before the first ELP amino acid. Briefly, a GST-ELP preparation on glutathione-affinity beads is washed several times in cleavage buffer (50 mM Tris HCl pH 7.0, 150 mM NaCl, 1 mM EDTA, 1 mM DTT). Thrombin is then added and the samples are incubated for over 16 hrs at room temperature. Supernatants are then collected and analyzed for successful cleavage of ELP from the beads by polyacrylamide gel electrophoresis and silver staining or Western blot. The purified proteins can be used e.g. to generate anti-ELP antibodies as described in (Harlow and Lane 1988).

Example VII: Protein-protein interactions involving ELP

An in vitro co-immunoprecipitation assay can be performed to find or confirm ELP interaction partners. For instance, HEK293 cells at 50% confluence are transfected by a lipofection method. For this purpose, mammalian expression vectors containing cDNA encoding for tagged ELP and potential interaction partners are combined with Lipofectamine transfection reagent (Life Technologies, Inc.) following the manufacturer recommendations, and overlaid onto monolayers of cells. Cells are lysed 25 hrs after transfection in co-IP buffer (20 mM Tris HCl pH 7.5, 140 mM NaCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM DTT, 1% Triton-X100, 10 % glycerol, 1 mM Natrium vanadate, 50 mM NaF, and protease inhibitors). Immunoprecipitations are performed in co-IP buffer using anti-tag antibodies (e.g. anti-HA, clone 3F10, Boehringer Mannheim) conjugated to protein G-agarose (Boehringer Mannheim). Control immunoprecipitations are performed using rat or mouse IgG (Sigma-Aldrich). After 3 hrs incubation at 4 °C, beads are washed 4 times in washing buffer (20 mM Tris HCl pH 7.5, 140 mM NaCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM DTT, 1% Triton-X100, 1 mM Natrium vanadate, 50 mM NaF) and resuspended in 25 µl of Laemmli buffer. Immune complexes are analyzed by SDS-PAGE/immunoblot assay using anti-ELP polyclonal antibodies provided by the invention or anti-tag antibodies, followed by horseradish peroxidase conjugated secondary antibody (Amersham Pharmacia Biotech). Detection can be performed using an enhanced chemiluminescence detection method (e.g. ECL, Amersham Pharmacia Biotech).

A GST-fusion protein in vitro binding assay can be performed e.g. to map binding domains, confirm an interaction partner or find additional interacting proteins. For this purpose, proteins are in vitro

translated (IVT) using reticulocyte lysates (TNT-lysates, Promega Corporation) containing [35S] methionine following the instructions provided by the manufacturer. Glutathione S-transferase (GST) fusion proteins, produced as illustrated in the Example VI, are immobilized on glutathione-Sepharose and blocked in binding buffer (20 mM Tris pH 8.0, 200 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM MgCl2, 10% glycerol, 0.5% NP40, 0.05% BSA, and proteinase inhibitors) for 45 min. Two µg of immobilized GST proteins are then incubated for 1.5 hrs with 0.5-4 µl of IVT proteins in binding buffer. The beads are washed four times in washing buffer (20 mM Tris pH 8.0, 200 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM MgCl2, 0.5% NP40) and boiled in Laemmli SDS sample buffer. The use of equivalent amounts of intact GST fusion proteins and successful IVT of the cDNAs have to be confirmed by SDS-PAGE analysis using Coomassie staining or autoradiography, respectively.

A yeast two hybrid assay can additionally be performed to confirm the results of the in vitro binding assays described above or to screen a cDNA library for new interaction partners (Fields and Sternglanz 1994). To confirm a specific binding or to map the binding region between ELP and an interaction partner, the desired cDNAs are subcloned into appropriate yeast expression vectors that link them either to a Lex DNA binding domain (e.g. pLexA, Clontech) or an acidic activation domain (e.g. pGJ4-5, Clontech). The appropriate pair of plasmids is then transformed together with a reporter plasmid (e.g. pSH18-34, Clontech) into an appropriate yeast strain (e.g. EGY48) by the lithium acetate-polyethylene glycol method and grown on selective media (Sambrook, Fritsch et al. 1989). Transformants are analyzed for reporter gene activity as described by the manufacturer of the vector-reporter plasmid used. To establish reproducibility the interactions is tested in both directions.

To isolate novel ELP-binding proteins (Bartel, Fields "The Yeast two-Hybrid System" Oxford UP, 1997) an appropriate yeast strain is transformed with a beta-Galactosidase reporter plasmid, a yeast expression vector containing ELP cDNA, or parts thereof, fused to the LexA DNA-binding domain sequence ("bait vector") and a second yeast expression vector containing a transcriptional activation domain fused to a collection of cDNA sequences ("prey vector" library, e.g. RFLY1 0-12 h embryo library, described in PNAS 93, 3011ff.). The triple transformants containing the reporter plasmid, and the bait and prey vectors are then grown on selective media, and selected for interaction-dependent activation of the auxotrophic and beta-Galactosidase reporters. From selected clones the respective prey construct is reisolated and the specificity of bait/prey-interaction is assessed, by checking for absence of interaction with unrelated bait-constructs. Finally the confirmed interactors are sequenced and full-length cDNAs are assembled and tested again for specific interaction with the bait.

Example VIII: Immunohistochemistry

Localization of ELP protein can be performed on Drosophila embryo, imaginal discs, adult tissue sections, vertebrate tumor cell lines, or vertebrate tissues using the anti-ELP antibodies provided by this invention. For instance, if a transformed cell line like HEK 293 cells (ATCC) is used, cells are seeded into polylysine-coated 8 well chambers (Nalge-Nunc Internat.) and grown overnight at 37ºC. The next day, cells are fixed with 3.7% formaldehyde in PBS for 10 min, permeabilized in 0.5% Triton-X-100 for another 10 min, and blocked with a 1:1000 dilution of pre-immunoserum in 2% BSA-PBS for 1 h at RT. Cells are then incubated with a

1: 2000 dilution of anti-ELP polyclonal rabbit immunoserum provided by this invention for 2 hrs at RT. The slides are washed three times for 5 min in PBS and incubated with a 1:200 dilution (v/v) of TRITC-conjugated swine anti-rabbit immunoglobulin (Dako, Inc.). The washing step is repeated before applying coverslips using Vectashield® mounting medium (Vector Laboratories, Inc.). As a positive control for specific staining part of the cells can be transfected e.g. by a lipofection method with a ELP expression plasmid, such as pcDNA3.1 (Invitrogen). Two days after transfection, control cells are stained with anti-ELP antibodies as described above.

Example IX: RNA interference experiments

RNA interference (RNAi) is a form of post-transcriptional gene silencing mediated by short double stranded RNAs (dsRNA) that has been described in plants, nematode, invertebrates organisms and mammalian cell culture ((Ngo, Tschudi et al. 1998; Vaucheret and Fagard 2001) (Kennerdell and Carthew 1998; Caplen, Fleenor et al. 2000; Elbashir, Harborth et al. 2001; Timmons, Court et al. 2001). DsRNAs have been shown to induce a degradation response in which single stranded RNA complementary to the short dsRNA is rapidly degraded (Montgomery and Fire 1998; Montgomery, Xu et al. 1998). RNAi can thus be used to reduce gene expression for instance in whole organisms or invertebrate and vertebrate cell lines (Kennerdell and Carthew 1998; Caplen, Fleenor et al. 2000; Clemens, Worby et al. 2000; Elbashir, Harborth et al. 2001). Several methods to introduce dsRNA into cells can be found in the literature. By hand of an example, we describe herein the treatment of Drosophila cells with delp dsRNA.

ELP dsRNA preparation

ELP dsRNA can be made from cDNA or genomic DNA templates, as long as most of the dsRNA corresponds to exon regions. Normally, target regions of 700 to 800 base pair are the most active. However, is known that dsRNAs as short as 200 base pair and as long as 2000 base pairs have potent interfering activities. Both RNA strands can be synthesized simultaneously from a PCR fragment, which contains for instance a T7, SP6 or a T3 promoter on each end. This PCR fragment can be generated by amplification of ELP cDNA or genomic DNA with 2 primers containing e.g. T7-polymerase binding sites. Primers complementary sequences should be 20 to 24 nucleotides in length with a 22 nucleotides optimum and 60°C optimum Tm. The 5' end of each primer should correspond to e.g. a 27 nucleotides T7 promoter sequence. The PCR reaction is then performed with a suitable template containing ELP sequences. Taq polymerase gives the best yields, but another polymerase like Pfu may be used, too. The first 10 cycles should have a 40°C annealing step, followed by 35 cycles with a 55°C annealing step. DMSO can be added to a final concentration of 5% when needed. Phenol-chloroform extract and ethanol precipitation in NH4OAc may be used to isolate the PCR template from the reaction mix however other commercially available PCR-purification kit can be used as well. The RNA synthesis reaction can be performed in 50 µl volume with 1 µg of PCR DNA template using an appropriate RNA polymerase. The MEGAscriptTM kits from Ambion work very well. The RNA becomes double-stranded during the synthesis reaction. The DNA template can be removed with RNase-free DNAase and the dsRNA can be purified by phenol-chloroform extraction and ethanol precipitation. Typical yields of RNA from 1 µg DNA template are in the 80 to 120 µg range. dsRNA is stored as a NaOAc/ethanol precipitate at -80°C until immediately before use.

The quality of the dsRNA can be monitored by native agarose gel electrophoresis in TBE. Only preparations should be used in which the electrophoretic mobility of most of the RNA is shifted to the mobility expected for dsRNA (very close to duplex DNA mobility) of the appropriate length.

Transfection of ELP dsRNA into Drosophila S2 cells

S2 cells are propagated in Schneider S2 Drosophila medium (GIBCO) supplemented with 10% FCS. One day before transfection one million cells are seeded into 6 well plates and grown overnight at 25°C. Cells are then transfected using the cationic lipid CellFectine (GIBCO) using an adaptation of the manufacturer's protocol. Briefly, a total of 5 µg DNA and dsRNA is complexed with 20 µl of CellFectine lipid mix in 1.2 ml serum free growth medium (e.g. DES expression medium from Invitrogen, Carlsbad,USA). The complexes are incubate for 15 minutes at RT and then added to the cells from which the normal growth medium has been replaced with 1 ml serum free medium. Four hours later 1.2 ml growth medium supplemented with 30 % FCS is added to the cells. One day after transfection the medium is replaced with fresh medium containing 10 % FCS. Cells can be assayed from 2 days after transfection (e.g. for ELP protein level or for Tcf transcriptional activity).

Similarly, mammalian ELP expression can be reduced using the method described in (Elbashir, Harborth et al. 2001).

Example X: Identification and cloning of an alternative help splicing variant and a chimeric est

Two human est isoforms of ELP were found in the public est database: a short and a long version. The short one lacks 54 bases close to the c-term, probably due to different or incomplete splicing (Figure 3). Both isoforms were cloned by standard molecular biology techniques using gene specific primes and est/cDNA clones (short: BE615647, BE61693, BG528377; long:cDNA clone AL529948).

hElp reside in 5q, a chromosomal region frequently deleted in some types of cancer (Genomics 2000 May 15;66(1):26-34). It is postulated that, the loss of genetic material from this chromosomal region in association with a specific syndrome is suggestive of a recessive mechanism of tumorigenesis, and that the deleted chromosome bands harbor a tumor suppressor. The growth inhibitory activity of ELP indicates that ELP might be the tumor suppressor in this region.

To support this hypothesis, the inventors found by complete sequencing of one of the hELP est ordered from the public database a chimeric est composed of the C-term of h-ELP fused to AF156165, a gene discovered by mapping the 5q chromosome region (Genomics 2000 May 15;66(1):26-34). h-ELP and AF156165 are located between the regions 5q23 and 5q33 and the 5q31 and 5q32, respectively. The chimeric est might have arisen from a deletion between the two genes and produce an incomplete ELP protein lacking the ENTH domain.

Example XI: ELP RNA expression in normal vs. tumor tissues

Cancer Profiling Array (BD Biosciences cat. 7841-1) membranes were hybridized with 5` labeled hELP probe (nucleotides 751 to 1951). Hybridization was

performed at 68°C, according to manufacturer's recommendation with a final washing using 0.2 x SCC and 0.5% SDS at 68°C for 15 min. For sample reference see BD bioscience catalog. A dot-blot showing ELP RNA expression in a set of normal versus tumor tissues is found in Figure 5 showing expression of hELP transcript and the percentage of tumors having increased and decreased ELP expression is summarized in Table 3 below.

Cancer type	number of sample	Decrease*	Same*	Increase *
Breast	50	36%	46%	18%
Uterus	42	19%	62%	19%
Colon	35	37%	60%	3%
Stomach	**27**	**52%**	**37%**	**11%**
Ovary	14	43%	36%	21%
Lung	**21**	**76%**	**24%**	**0%**
Kidney	**20**	**60%**	**35%**	**5%**
Rectum	18	16%	78%	5%
Thyroid	6	33%	50%	17%
Prostate	4	0%	100%	0%
Pancreas	1	100%	0%	0%
Cervix	1	0%	100%	0%
Small Intestine	2	50%	50%	0%

* Tumor compared to normal tissue

Table 3. Summary of the data of Figure 5.

Reductions in hELP mRNA expression compared with their respective normal tissues was observed in the majorities of lung, kidney and stomach cancer samples. The ELP RNA down regulation was found in around 3/4 of all lung cancers and over 1/2 of kidney and stomach cancer samples (indication of a potential tumor supression function: "tumors want to get rid of it").

While there are shown and described presently preferred embodiments of the invention, it is to be distinctly understood that the invention is not limited

thereto but may be otherwise variously embodied and practiced within the scope of the following claims.

The disclosure of all literture/publications cited throughout the specification is incorporated herein by reference in its entirety.

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